Implication of RANTES in the Modulation of Alloimmune Response By Progesterone During Pregnancy

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Problem
Several studies indicate that RANTES (regulated on activation, normal T cell expressed and secreted) is able to downregulate T-cell responses which suggest it might be relevant for fetal tolerance induction. However, the role of RANTES in pregnancy had not been established. Here we investigate RANTES regulation during early pregnancy and potential failures leading to losses of pregnancies.

Method of study
RANTES and progesterone levels were determined in sera and feto-placental units from high resorption rate CBA/J × DBA/2 pregnant females and compared with CBA/J × BALB/c normal pregnant mice. RANTES *in vitro* modulation was also studied in nulliparous, primiparous and multiparous CBA/J and BALB/c cells in response to paternal alloantigen and progesterone stimulation.

Results
Nulliparous CBA/J females were quantitatively deficient in RANTES sera levels, whereas pregnancies with male BALB/c or DBA/2 increased its production. However, feto-placental units from CBA/J females are high producers of progesterone and RANTES.

Conclusion
These data suggest that the beneficial effect of RANTES on feto-maternal interface requires an optimal concentration range and might be modulated by progesterone, hence exacerbated placental expression could be associated with high resorption rate.

Introduction

Immune cells and their secretory products have been recognized as important mediators of the pathophysiology of recurrent spontaneous abortions (RSA) and endometriosis.[1–5] On the other hand, chemokines are involved in T-cell trafficking during normal processes but also in pathological events such as inflammation and endothelium damage.[6]

In this sense, RANTES (regulated on activation, normal T cell expressed and secreted) is a chemokine recently implicated in trophoblast and spermatozoa migration because of their well-established chemoattractant properties.[7,8] However, instead of its specific ability to downregulate T-cell responses which suggests it might be relevant for fetal tolerance induction, the role of RANTES in pregnancy had not been established.

We recently demonstrated that serum RANTES is diminished in patients with RSA and that immunization with paternal leukocytes normalized these levels. Moreover, RANTES serum levels in women with

no previous pregnancies or abortions were also significantly lower compared with fertile women, suggesting that successful pregnancy is accompanied by an increase in RANTES serum production.[9]

On the other hand, it has been demonstrated that RANTES concentration and bioactivity are elevated in peritoneal fluid of women with endometriosis.[10,11] Moreover, clinical effectiveness of chronic progestin treatment in endometriosis-associated pelvic pain was attributed to inhibition of RANTES gene transcription. This effect was progesterone dependent and mediated by nuclear factor-κB pathway.[5] Taking into account that implantation window is considered an inflammatory process which needs to be followed by a Th2 shift in order to control endocrine and immune system,[12,13] and that progesterone stimulates Th2 response and diminishes inflammatory cytokines,[14,15] we hypothesized that failure in RANTES regulation might lead to pregnancy loss.

In this sense, the preeminent mice model to study immunological RSA is the mating CBA/J female to DBA/2 male combination.[16] This results in partner-specific high number of genetically normal resorptions. This reproductive model involved a sequence of events leading to abortions as the production of proinflammatory cytokines such interleukin-1, tumor necrosis factor-α, interferon-γ. Considering that high levels of fetal losses in CBA/J $\times$ DBA/2 murine model is also associated with an exacerbated inflammatory response,[16] the aim of this work was to evaluate RANTES production levels in sera and placentas of this abortion combination and its possible modulation by progesterone.

Material and methods

Mice

CBA/J female mice were from the Animal Center of the Atomic Energy National Argentinean Commission. Male DBA/2 and BALB/c mice were obtained from the Animal Center of the Immunology Department, School of Pharmacy and Biochemistry, University of Buenos Aires. Animals were held in a clean-cage animal facility on a 12 hr light/dark cycle and maintained under conventional open-top wire cage conditions with food and water *ad libitum*. Female CBA/J and BALB/c mice were mated by overnight cohabitation with a DBA/2 male. Morning of vaginal plug sighting was defined as 0.5 day of pregnancy.

Feto-Placental Culture Supernatants

Mice were killed on pregnancy day 10.5. Uteri were removed and feto-placental units were mechanically disrupted and cultured in 10% fetal bovine serum, glutamine and penicillin–streptomycin supplemented RPMI 1640 (Gibco, Los Angeles, CA, USA). Incubation was set up in a 5% CO_2 humid atmosphere at 37°C, for 48 hr. Supernatants were collected and stored at −70°C for further analysis.

RANTES Quantification

RANTES was assayed in sera and feto-placental culture supernatants from CBA/J and BALB/c nulliparous, primiparous and multiparous females with commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D System, Minneapolis, MN, USA; Life Technologies, Grand Island, NY, USA). ELISA test was performed according to the manufacturer's instructions and results were expressed as pg/mL.

Progesterone Quantification

Progesterone levels on feto-placental culture supernatants were determined by competitive chemiluminescence assays using Access auto analyzer (Beckman Lab., Fullerton, CA, USA). Analytical sensitivity was 0.08 ng/mL.

Mixed Lymphocyte Reaction (MLR)

Spleen cells obtained from CBA/J and BALB/c females and DBA males, extensively washed and resuspended in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum, glutamine and penicillin–streptomycin. Mixture of stimulatory leukocytes, treated with mitomycin C (1×10^5 cells/well) and responder cells (1×10^5 cells/well) were incubated in a 96-well U-shape microtiter plate (Becton Dikinson, Franklin Lakes, NJ, USA) in the absence or presence of progesterone 10^{-5} M (Sigma, St Louis, MO, USA). After 3 days in culture at 37°C in a 5% CO_2 atmosphere, supernatants were collected and RANTES secretion was measured by ELISA.

Statistical Analysis

One-way ANOVA, Student's *t*-test and Newman–Keuls multiple comparison test were performed when appropriate.

Results

Modulation of RANTES During Pregnancy

Considering data reported previously showing that both RSA patients and nulliparous women have significantly decreased RANTES sera levels in comparison with fertile women,[9] we studied sera levels of this chemokine in nulliparous CBA/J females with regard to BALB/c. Fig. 1 shows that RANTES levels in CBA/J sera was significantly diminished with regard to BALB/c (48.34 ± 12.2 versus 247.5 ± 114.9 pg/mL, respectively $P < 0.05$ Student's *t*-test). On the other hand, as RSA patients have the ability to increase RANTES systemic levels after stimulation with paternal leukocytes,[9] we investigated differences in serum RANTES levels after multiparous pregnancies with BALB/c or DBA/2 males. As depicted in Fig. 1, RANTES sera production in multiparous CBA/J females was significantly increased after mating with BALB/c and also DBA/2 males with regard to nulliparous CBA/J levels (443.8 ± 103.6 and 238.8 ± 43.05 pg/mL, respectively; $P < 0.001$ Student's *t*-test).

In vitro Modulation of RANTES by Progesterone

Considering that RANTES production was modulated by paternal stimulation and that its gene transcription in women with endometriosis was downregulated by prolonged exposure to progestin,[5] we investigated the ability of progesterone to modulate RANTES secretion during alloantigen response in an MLR. Spleen lymphocytes obtained from CBA/J and BALB/c females were assayed in an MLR performed with DBA/2 spleen cells in response to different progesterone concentrations ranging from 10^{-2} to 10^{-5} M. After 3 days of allogeneic response, supernatants were collected and RANTES secretion was measured by ELISA. Fig. 2 shows that CBA/J lymphocytes in response to 10^{-5} M of progesterone (physiological concentration reported at the feto-placental unit) were able to significantly increase RANTES secretion ($P < 0.05$ Student's *t*-test).

Differential Progesterone Production in Abortion

Taking into account that fetal losses in CBA/J × DBA/2 mice occur during placental vascularization window[17] and considering the hypothesis that progesterone could modulate RANTES levels, we decided to investigate progesterone production in day 10.5 placental culture supernatants from normal and abortion combination. Fig. 3 shows that placentas from primiparous high resorption rate CBA/J × DBA/2 pregnancies, displayed higher progesterone production in comparison with non-abortive combination, CBA/J × BALB/c (6.14 ± 0.54 versus 4.54 ± 0.34 pg/mL; $P < 0.001$ Student's *t*-test). Whereas, placentas from multiparous CBA/J females mated with DBA/2 or BALB/c males which exhibit less abortion rate, produced less progesterone with

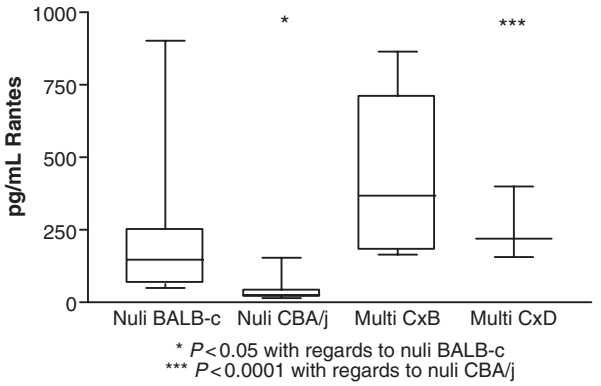

Fig. 1 Sera levels of RANTES were determined by enzyme-linked immunosorbent assay. Results are expressed as the mean (pg/mL) from eight BALB/c and CBA/J nulliparous females, and eight CBA/J × DBA/2 and CBA/J × BALB/c multiparous females. CBA/J nulliparous females showed a significant decrease in RANTES sera levels compared with BALB/c nulliparous females (*$P < 0.05$, Student's *t*-test). RANTES sera levels production increase after multiples pregnancies (**$P < 0.0001$ Student's *t*-test).

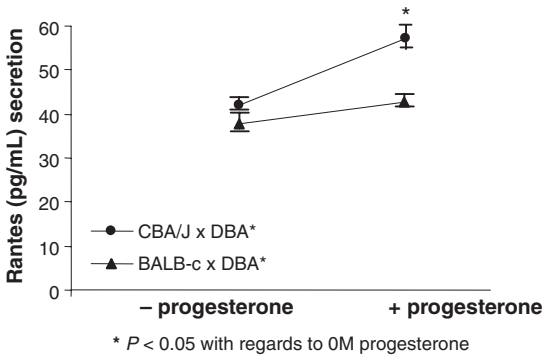

Fig. 2 Mixed lymphocyte reactions were performed with CBA/J or BALB/c female spleen lymphocytes stimulated with male DBA/J spleen lymphocytes. Results are representative out of three independent experiments using different mice and expressed as mean (pg/mL). Progesterone increased CBA/J female lymphocytes RANTES secretion in response to DBA/2-allogeneic stimulation (*$P < 0.05$, Student's *t*-test).

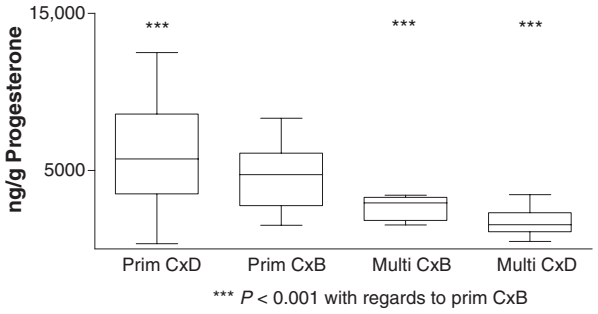

Fig. 3 Feto-placental units were obtained from CBA/J × DBA/2 and CBA/J × BALB/c combinations at 10.5 days of pregnancy. Results are expressed as the mean (ng/g) of six mice from each group. CBA/J primiparous females produce significantly higher progesterone levels (***P < 0.001, Student's t-test).

regard to primiparous (1.67 ± 0.22 and 2.62 ± 0.35 pg/mL, respectively; $P < 0.001$ Student's t-test).

Differential RANTES Production in Abortion

Considering that progesterone is increased in placentas from abortion combination CBA/J × DBA/2 and diminishes with multiparity, and taking into account that abortion rate is decreased in multiparous CBA/J × DBA/2 pregnancies[18] we assayed RANTES secretion in CBA/J primiparous and multiparous females mated with DBA/2 and BALB/c males. The recovered feto-placental units were cultured during 72 hr and RANTES secretion quantified in the collected supernatants. Reassembling results obtained in sera, placentas from multiparous CBA/J × DBA/2 displayed significantly lower RANTES production in comparison with the control combination (1670 ± 217 versus 2620 ± 350 pg/g; $P < 0.05$ Student's t-test) (Fig. 4a). However, placentas from primiparous abortion combination CBA/J × DBA/2 displayed significantly higher RANTES levels with regard to placentas from normal combination (2970 ± 260 versus 2270 ± 170 pg/mL; $P < 0.001$ Student's t-test) (Fig. 4b).

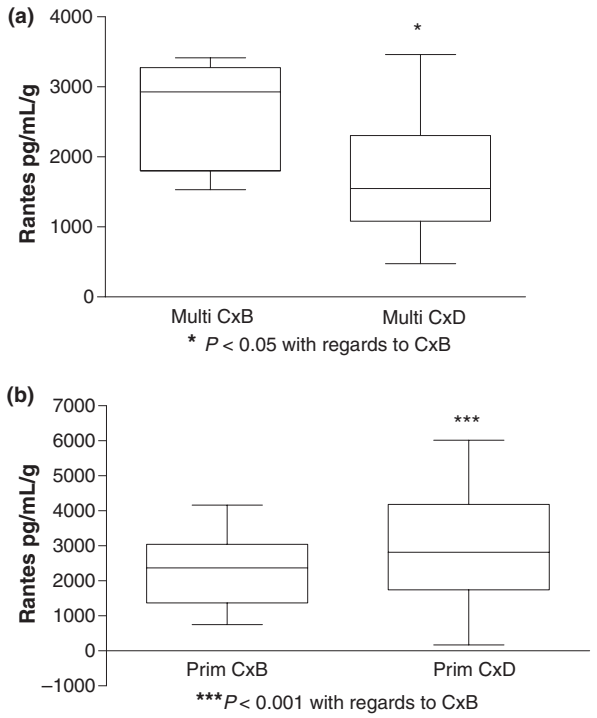

Fig. 4 RANTES production at the feto-maternal interface. Feto-placental units were obtained at 10.5 days of pregnancy from multiparous CBA/J (a) or primiparous (b) mouse mated with DBA/2 and BALB/c males. Results are expressed as the mean (pg/mL) of six subjects from each group. Multiparous CBA/J females secreted significantly lower RANTES levels in comparison with BALB/c females (*P < 0.05, Student's t-test). In contrast primiparous CBA/J females produced significantly higher RANTES levels in comparison with respective control mated combination (***P < 0.001, Student's t-test).

Discussion

Feto-maternal tolerance is an active process that employs evolutionarily conserved mechanisms such as apoptosis, anergy, or T-cell regulation.[18,19] Tolerance of alloreactive lymphocytes induced by RANTES might represent one of the mechanisms involved in fetal-semiallograft survival. Recent studies demonstrates that RANTES participates in the generation of peripheral tolerance by inducing $CD8^+$ T regulatory cells[20] and its *in vivo* blockade prevent the generation of $CD8^+$ T regulatory cells.[20–22]

High levels of progesterone and alloactivation by paternal alloantigens are two main actors that participate during and after the process of implantation. Challenge with alloantigens that occurs during single or multiple pregnancies, increased RANTES serum levels. This effect was previously observed in fertile women along normal pregnancies but also was induced in RSA patients after paternal immunization.[10,24]

In the present study, we found that nulliparous CBA/J females were quantitatively deficient in RANTES sera levels, whereas pregnancies with male BALB/c or DBA/2 increased its production. Similar to the human counterpart, the high resorption rate of CBA/J × DBA/2 combinations could be explained,

at least in part, by the presence of low serum level of RANTES.

However, although inflammatory response by RANTES expression seems to be needed for a successful implantation, exacerbated levels in feto-maternal interface could be associated with pathological conditions like endometriosis.[13] Results obtained in this study indicate that feto-placental units from CBA/J females are high producers of progesterone and RANTES. Taken together, these data suggest that the beneficial effect of RANTES on feto-maternal interface requires a modulation of optimal concentration range. We also suggest that progesterone could be implicated in this modulation. Previous *in vitro* studies from our lab could explain this apparent paradox. We found that recombinant RANTES was able to suppress the allogeneic response when used in doses that ranged between 30 pg/mL and 1 ng/mL. However, inhibition of the allogeneic response was not observed when recombinant RANTES (rRANTES) was used at 10 ng/mL.[10] Differential effects of medium or high levels of RANTES in the endometrium, could be a potential explanation for the final outcome toward a pregnancy success or the development of conditions like abortions or endometriosis.

In this context, we could postulate that placentas from primiparous CBA/J females are high producers of progesterone that could induce high levels of RANTES correlating with a potent exacerbated Th1 response. This deleterious effect was abrogated after multiple pregnancies.

The specific ability of a given range of RANTES concentration levels to downregulate T-cell responses and to be modulated by progesterone and alloantigens suggests that it might be relevant for fetal tolerance induction and could be potentially used to avoid recurrent miscarriage. The present data provide additional new insight for the close relationship between immune and endocrine systems both for normal and abnormal conditions. Investigation of the molecular mechanisms leading to immune tolerance and homeostasis will contribute to delineate novel therapeutic strategies to prevent immunologically mediated failures.

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